Proteolytic Activities of Some Oral Spirochetes
Robert R. Omata and Edward G. Hampp

J DENT RES 1961 40: 171
DOI: 10.1177/00220345610400010901

The online version of this article can be found at:
http://jdr.sagepub.com/content/40/1/171

Published by:
SAGE
http://www.sagepublications.com
On behalf of:
International and American Associations for Dental Research

Additional services and information for Journal of Dental Research can be found at:

Email Alerts: http://jdr.sagepub.com/cgi/alerts
Subscriptions: http://jdr.sagepub.com/subscriptions
Reprints: http://www.sagepub.com/journalsReprints.nav
Permissions: http://www.sagepub.com/journalsPermissions.nav
Citations: http://jdr.sagepub.com/content/40/1/171.refs.html

>> Version of Record - Jan 1, 1961
What is This?
Proteolytic Activities of Some Oral Spirochetes

ROBERT R. OMATA and EDWARD G. HAMPP*

National Institute of Dental Research, National Institutes of Health
Bethesda, Maryland

Few studies on the metabolism of spirochetes have been published. Chang reported the non-utilization of sugars and the requirement of serum for growth by *Leptospira icterohaemorrhagiae*. Marshall and Helprin and Hiatt studied the stimulatory effect of serum and fatty acids on the respiration of leptospiroae, while Fulton and Spooner, using *L. icterohaemorrhagiae*, analyzed the growth medium for chemical changes and found that accumulations of small quantities of fatty acids occurred and that there was no significant disappearance of protein during growth. Barban reported transamination by the Reiter treponeme in the formation of glutamic acid, aspartic acid, alanine, and glycine. Markovetz and Larson demonstrated transamination by *L. biflexa*, with pyridoxal phosphate as the coenzyme, in the formation of glutamic acid, aspartic acid, and alanine. Berger reported the dissolution of the "mucin clot" from synovial fluid by culture filtrates of certain oral spirochetes, although Hampp, Mergenhagen, and Omata could demonstrate no mucopolysaccharase activity by oral spirochetes. Recently the nutritional requirements of the oral treponemes and *Borrelia vincentii* were studied extensively by Hampp and Nevin and a partially defined medium which contained only small amounts of ascitic fluid and several growth factors was devised.

Several preliminary experiments were performed in connection with earlier work on the enzymic activities of oral spirochetes (unpublished data) which indicated proteolytic activity of these micro-organisms. The present report concerns the utilization of protein by strains of oral treponemes and of *B. vincentii* in relation to the phases of their growth.

MATERIALS AND METHODS

The basal medium employed has been described previously as essentially a veal-heart infusion prepared in a sterilizer and containing thiopeptone (BBL). At the time of use, appropriate quantities of the medium were heated in a boiling-water bath to remove residual air, then enriched with 0.1 per cent reduced glutathione and canine ascitic fluid as required. The latter substance is considered to be indispensable for spirochetal growth; however, by gradual reduction of the added ascitic fluid over an extended number of transfers it was possible to adapt two strains of oral spirochetes to grow in the basal medium without it. The two primary strains used, FM, a small oral treponeme, and N9, *B. vincentii*, were maintained on the basal medium enriched

Received for publication May 31, 1960.

* Senior Research Associate, American Dental Association at the National Institute of Dental Research.
with 0.1 per cent reduced glutathione and 1 per cent canine ascitic fluid (AF medium). The two adapted variants FM-G and N9-G, were maintained on the basal medium supplemented with glutathione only (G medium).

In the experiment, multiple tubes, 15 × 125 mm., containing 14 ml. of the respective media, were each inoculated with 1 ml. of a 7-day culture of the respective strain. Uninoculated tubes were included as controls. All tubes were closed with sterile rubber stoppers to exclude air and incubated at 35°C.

At intervals of 2–21 days, duplicate tubes of each culture and of its uninoculated controls were removed for analysis. The number of spirochetes was determined by direct counting of suitable dilutions in a Petroff-Hauser chamber. Each count was the average of at least four determinations.

Five ml. from each culture tube and control tube were centrifuged at 5°C to remove spirochetes and suspended protein of the medium. Half of each supernatant was treated with an equal volume of 10 per cent trichloracetic acid (TCA) and centrifuged at 5°C to remove precipitated proteins. "Protein values" were determined on all supernatants by the method of Lowry, Rosebrough, Farr, and Randall.13 The true protein content of each tube was considered to be the difference between the protein value of the untreated supernatant and the protein value of the corresponding TCA-treated supernatant. These results were substantiated in pilot experiments by direct determination of the protein values of the TCA-precipitated protein. The average protein contents of the uninoculated controls were 3.1 mg/ml for the G medium and 3.7 mg/ml for the AF medium.

In addition, amino acid values were determined on the TCA-treated supernatants by the procedure of Rosen.14 The results on the uninoculated controls (G and AF media) were strikingly similar, ranging from 5.9 to 6.5 mg. of leucine equivalents per milliliter.

RESULTS

The data presented in Table 1 are the results of the proteolytic activity of the oral spirochetes correlated with cell counts at various time intervals. Since the degree of proteolytic activity of the various strains of micro-organisms up to the seventh day was related to the number of cells present at the time of analysis of the culture supernatants and since some variations were observed at later periods, it is expedient to discuss the characteristics of their growth phases. At the time of inoculation of the cultures, the initial cell counts for B. vincentii N9 and its variant N9-G were 13.3 × 10^9, and the small oral treponeme FM and its variant FM-G were 24 × 10^9 cells per milliliter.

The growth phases of both strains of B. vincentii in their respective media did not differ greatly from the second through the fourteenth days. The logarithmic phases in both instances occurred between the second and seventh days, and the points of stationary phase were reached by the fourteenth day. By the twenty-first day, it was difficult to count the cells in the G medium because of the formation of spirochetal granules and some loss of cellular integrity, resulting in lower total cell counts. On the contrary, however, successful enumeration of cells in the AF medium was possible because the organisms retained their morphologic characteristics, with only limited granule formation, and showed a slight increase in the total number of cells, as evidenced in the table.
The proteolytic activity of the two strains of *B. vincentii* (N9) and its variant (N9-G) was not manifested during the second day of growth; on the contrary, there were slight increases in the protein values in both the AF and the G media. An explanation for the observation is not available and is seemingly not consistent with an error in protein analysis. However, by the fourth day, medium AF showed a decrease in protein, whereas the G medium still retained an increased protein value, although it was roughly half that for the preceding time interval. During this period, however, there were increased amino acid values, as indicated in Table 1. When the peak of the logarithmic phase was attained on the seventh day by either strain of spirochetes in

**TABLE 1**

<table>
<thead>
<tr>
<th>DAYS</th>
<th>AV. CELL COUNT N×10⁹/ML</th>
<th>PER CENT CHANGE IN PROTEIN</th>
<th>PER CENT CHANGE IN AMINO ACIDS</th>
<th>AV. CELL COUNT N×10⁹/ML</th>
<th>PER CENT CHANGE IN PROTEIN</th>
<th>PER CENT CHANGE IN AMINO ACIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.3</td>
<td>+11.9</td>
<td>+6.7</td>
<td>13.3</td>
<td>+17</td>
<td>+9.5</td>
</tr>
<tr>
<td>2</td>
<td>67.5</td>
<td>-2.9</td>
<td>+3.1</td>
<td>67.5</td>
<td>-47.5</td>
<td>+22.4</td>
</tr>
<tr>
<td>4</td>
<td>177.5</td>
<td>-12.5</td>
<td>+28.9</td>
<td>170</td>
<td>-18.3</td>
<td>+54.5</td>
</tr>
<tr>
<td>7</td>
<td>220</td>
<td>-25.7</td>
<td>+17.5</td>
<td>237.5</td>
<td>-47.5</td>
<td>+22.4</td>
</tr>
<tr>
<td>14</td>
<td>287</td>
<td>-12.5</td>
<td>+28.9</td>
<td>283.5</td>
<td>-18.3</td>
<td>+54.5</td>
</tr>
<tr>
<td>21</td>
<td>313</td>
<td>-6.4</td>
<td>+58.1</td>
<td>180</td>
<td>+112.8</td>
<td>+59.5</td>
</tr>
</tbody>
</table>

Small Oral Treponeme

<table>
<thead>
<tr>
<th>DAYS</th>
<th>AV. CELL COUNT N×10⁹/ML</th>
<th>PER CENT CHANGE IN PROTEIN</th>
<th>PER CENT CHANGE IN AMINO ACIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>+45.2</td>
<td>+20</td>
</tr>
<tr>
<td>2</td>
<td>270</td>
<td>+48.1</td>
<td>+57.7</td>
</tr>
<tr>
<td>4</td>
<td>530</td>
<td>-95.1</td>
<td>+95.1</td>
</tr>
<tr>
<td>7</td>
<td>700</td>
<td>-66.2</td>
<td>+96.9</td>
</tr>
<tr>
<td>14</td>
<td>731.5</td>
<td>+46.4</td>
<td>+111.6</td>
</tr>
<tr>
<td>21</td>
<td>714</td>
<td>+45.2</td>
<td>+26.2</td>
</tr>
</tbody>
</table>

Small Oral Treponeme

<table>
<thead>
<tr>
<th>DAYS</th>
<th>AV. CELL COUNT N×10⁹/ML</th>
<th>PER CENT CHANGE IN PROTEIN</th>
<th>PER CENT CHANGE IN AMINO ACIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>-19</td>
<td>+6.7</td>
</tr>
<tr>
<td>2</td>
<td>262.5</td>
<td>-44.4</td>
<td>+45.6</td>
</tr>
<tr>
<td>4</td>
<td>502.5</td>
<td>-65.2</td>
<td>+93.6</td>
</tr>
<tr>
<td>7</td>
<td>721</td>
<td>-63.8</td>
<td>+82.3</td>
</tr>
<tr>
<td>14</td>
<td>697.5</td>
<td>+52.5</td>
<td>+140.5</td>
</tr>
<tr>
<td>21</td>
<td>581</td>
<td>+52.5</td>
<td>+140.5</td>
</tr>
</tbody>
</table>

* + = percentage increase in protein and amino acid contents of the culture media; − = percentage decrease in protein contents of the culture media.

their respective media, the greatest percentage decrease in protein content occurred. Concomitantly, there were increased amounts of amino acids in both instances. By the fourteenth day, a declining proteolytic activity was evident, although the amino acid values continued to increase. On the twenty-first day, the AF culture showed a slight decrease in proteolysis and a twofold increase in the amino acid values compared with the preceding period; the former observation may possibly be due to the organisms reaching the stationary growth phase. However, the G culture medium showed only a slight increase in the amino acids, but the protein content was markedly increased. This change may possibly be associated with the low cell count, excessive granule formation, and loss of cellular integrity with autolysis, as previously indicated for this period of growth.

The small oral treponemes (FM and FM-G) in their respective media generally followed the growth phases of the *Borrelia* strains; however, the total cell counts were
much greater at any given time period (Table 1). The logarithmic phases of growth in AF and G media occurred between the second and seventh days, and the total cell counts in both instances were comparable. The points of stationary phase were reached during the seventh and fourteenth days. Declining cell counts were observed in the G medium by the fourteenth day, and at the twenty-first day marked changes occurred in cellular morphology associated with excessive granule formation, as was also observed with B. vincentii (N9-G).

The small oral treponemes showed greater proteolytic activity in their respective media at the selected time intervals than did the strains of B. vincentii. Proteolysis was evident as early as the second day in both the AF and the G media and was greater in the former; however, the amino acid values were comparable. The proteolysis in both media progressed at an increased rate during the logarithmic phase of growth and reached the peak of activity on the seventh day, which is also indicated by the increased amounts of amino acids in both culture supernates. From the seventh to the fourteenth days, the amino acid values remained constant in both cultures; however, the AF broth showed decreased proteolytic activity, and the G medium remained essentially unchanged. At the twenty-first day, both AF and G cultures showed declining proteolysis; however, the amino acid values were markedly increased in the G medium and to a lesser degree in the AF medium.

**DISCUSSION**

Ascitic fluid or serum supplements have been shown to be essential components for the isolation and cultivation of the family Treponemataceae. However, preliminary experiments indicated that the usual 10 per cent amounts of ascitic fluid employed for culturing these organisms complicated the protein and amino acid analyses. Therefore, eventually both parent strains of spirochetes (FM and N9) were successfully grown in the basic medium with reduced amounts of ascitic fluid (1 per cent). After numerous transfers, these organisms were eventually adapted to grow without ascitic fluid (FM-G and N9-G). The cell crops for all strains were comparable to those obtained with 10 per cent ascitic fluid in the basic medium. The mechanism of the adaptive process in the present study is not clear. Hampp and Nevin showed that the parent strains could be grown in the heat-altered basal medium, provided that additional growth factors were added to substitute for heat-labile constituents of the medium and ascitic fluid.

The demonstration of proteolytic activity by oral spirochetes supplies additional information on the metabolic activity of this group of micro-organisms. The differences in the proteolysis of the *Borrelia* strains and the strains of oral treponemes indicate variations in their nitrogen metabolism. Strains N9 and N9-G appear capable of using nitrogen-containing substrates smaller than the TCA-precipitable proteins—that is, TCA-soluble compounds, possibly peptides and polypeptides, which are provided in the culture media by thiopeptone and the veal-heart infusion. The small net change in the protein values and the smaller percentage of liberation of amino acids by the *Borrelia* strains suggest the elaboration of peptidases by these organisms. The small oral treponemes, FM and FM-G, showed greater proteolytic activity than did N-9 and N9-G strains by degrading greater amounts of TCA-precipitable components or the larger protein molecules, which are mainly provided in the media by the veal-heart in-
fusion and ascitic fluid. The “proteinase” activity of the treponemes was indicated by the greater diminution of proteins and the greater over-all increase in the liberation of amino acids over the growth range.

Diminished proteolytic activity of all strains of organisms commenced during the stationary phase of growth and was more manifest in the G medium. At the 21-day interval, the formation of spirochetal granules, the loss of cellular integrity, and the lower cell counts may in part be due to the diminished amounts of oleic and other fatty acids in the G medium in contrast to AF medium. Evidence was shown by Nevin and Hampp\(^{10}\) that in the absence of added oleic acid in their partially defined medium there were greater numbers of granules formed by these spirochetes.

**SUMMARY**

The proteolytic activities during the growth phase of two strains of *B. vincentii* and two of a small oral treponeme were demonstrated. The parent strain of *B. vincentii* (N9) and the adapted variant (N9-G), which was adapted to grow in the absence of ascitic fluid, grew equally well in their respective media and showed evidence of significant proteolytic activity. Cultures of the two strains of the small oral treponeme (FM and FM-G) showed greater proteolytic activity than did the *B. vincentii* strains.

The greatest degree of proteolysis by all strains was observed to coincide with the logarithmic growth phases, occurring during the second through the seventh days. During the stationary phases, there were diminished activities, especially with the *Borrelia* strains.

**REFERENCES**

16. KAST, C. C., and KOLMER, J. A. Methods for the Isolation and Cultivation of Treponemes with Special Reference to Culture Media, Am. J. Syphilis, Gonorrhea, Venereal Diseases, 24:671, 1940.